

PURIFICATION OF A LIPOPROTEIN LIPASE-INHIBITING PROTEIN PRODUCED BY A  
MELANOMA CELL LINE ASSOCIATED WITH CANCER CACHEXIA

Mitsuo Mori,<sup>1\*</sup> Ken Yamaguchi<sup>1</sup> and Kaoru Abe<sup>2</sup>

<sup>1</sup>Growth Factor Division, National Cancer Center Research Institute, Tokyo, Japan

<sup>2</sup>Department of Medicine, National Cancer Center Hospital, Tokyo, Japan

Received March 20, 1989

**SUMMARY:** A human melanoma cell line, SEKI, induces severe cachexia in tumor-bearing nude mice. A factor with the ability to inhibit lipoprotein lipase (LPL) was isolated from the conditioned medium of this cell line. This factor was 40-K-dalton protein, and designated temporarily as melanoma-derived LPL inhibitor (MLPLI). Amino acid sequencing revealed that the amino-terminal portion consists of SPLPITPV-AT--IR-P. Unexpectedly, the sequence, as far as determined, was identical to those of leukemia inhibitory factor (LIF), suggesting that MLPLI is a protein closely related to LIF. The findings that MLPLI inhibits LPL activity and that MLPLI is produced by human cancer cells inducing cancer cachexia also suggest that this protein is a candidate for the factor responsible for cancer cachexia.

© 1989 Academic Press, Inc.

Cancer cachexia is a morbidity that develops in animals or humans that bear tumors. Although the mechanisms responsible for this morbidity are poorly understood, the derangement in lipid metabolism is now believed to be one of the causes (1). In this context, many researchers have focused their attention to lipoprotein lipase (LPL) present in adipocytes; the suppression of this enzyme activity may lower the intake of fatty acids by adipocytes, resulting lipid catabolism in adipose tissues (2). Several factors with LPL-inhibiting activity are now known to be produced by the host cells in response to the presence of tumors. Tumor necrosis factor (TNF)- $\alpha$  (3), interleukin-1 (4) and interferon- $\gamma$  (5) are their representatives, and may participate in inducing cancer cachexia. However, there is no previous report indicating that these factors with LPL-inhibiting activity were isolated from tumor cells that induce cancer cachexia in tumor-bearing animals. In the present study, we isolated a factor with LPL-inhibiting activity from the medium conditioned by a melanoma cell line, SEKI, which induces severe cachexia in tumor-bearing nude mice.

\* To whom correspondence should be addressed.

0006-291X/89 \$1.50

Copyright © 1989 by Academic Press, Inc.

All rights of reproduction in any form reserved.

### Materials and Methods

**Reagents:** RPMI 1640, Dulbecco's modified Eagle's medium (DMEM), Dulbecco's PBS(-) and glutamine were obtained from Nissui Seiyaku Co. (Tokyo, Japan); donor calf bovine serum (DCBS) from Flow Laboratories (North Ryde, Australia); fetal calf serum (FCS) from Boehringer GmbH (Mannheim, W. Germany); dexamethasone, insulin and sodium heparin from Sigma Chemical Co. (St. Louis, MO, USA); penicillin and streptomycin from GIBCO (Grand Island, NY, USA); sodium bicarbonate from Ootsuka Seiyaku Co. (Tokyo); and methylisobutylxanthine from Aldrich Chemical Co., Inc. (Milwaukee, WI, USA). Glycerol tri[1-<sup>14</sup>C]oleate and [1-<sup>14</sup>C]oleic acid were purchased from Amersham (Buckinghamshire, England).

**Cells and Cell Culture:** 3T3-L1 preadipocytes provided by the Japanese Cancer Research Resources Bank (Tokyo) were cultured in 60-mm culture dishes in DMEM supplemented with 10% heat-inactivated (56°C for 30 min) DCBS, streptomycin (200 mg/ml), penicillin (100 units/ml), sodium bicarbonate (0.14%) and glutamine (0.584 mg/ml) in a humidified atmosphere of 5% CO<sub>2</sub>/95% air at 37°C. Differentiation was induced by the method of Rubin (6) with slight modifications. When 3T3-L1 cells were grown to confluence, the medium was changed to DMEM supplemented with 10% FCS, 10 µg of insulin per ml, 1 µM dexamethasone, and 0.5 mM methylisobutylxanthine. After incubation for 48 hours, the medium was changed to the original medium. Seven to 10 days after confluence occurred, the cells were used for the experiments. SEKI cell line (7) was established at the National Cancer Center (Tokyo). SEKI cells were cultured in RPMI 1640 medium supplemented with 5% heat-inactivated FCS, glutamine (0.3 mg/ml), penicillin (100 units/ml), streptomycin (200 mg/ml) and sodium bicarbonate (0.14%) in a humidified atmosphere of 5% CO<sub>2</sub>/95% air at 37°C in 75-cm<sup>2</sup> plastic tissue culture flasks.

**Preparation of Conditioned Medium of SEKI Cells:** To obtain serum-free medium, SEKI cells were washed twice with serum-free RPMI 1640 medium and suspended in the same medium, and then were seeded into 75-cm<sup>2</sup> plastic tissue culture flasks, each containing 45 ml of the medium. After 3 to 4 days of incubation at 37°C, the medium was collected aseptically, freed of cell debris and stored at -20°C.

**Tumor Xenograft in Nude Mice:** SEKI cell xenografts in nude mice were obtained by inoculation of cultured SEKI cells into the flank region of the animals subcutaneously. The specimens, about 5 x 5 mm<sup>2</sup>, from this xenograft were further transplanted into the flank region of nude mice subcutaneously.

**Assay of LPL Activity:** The test samples were dissolved in fresh medium described above, and then 3.6 ml of the sample solution was added to the differentiated 3T3-L1 cells. After exposure to the sample solution for 16 hours, the cells were washed twice with Dulbecco's PBS(-) and then incubated for further 60 minutes at 37°C with 1 ml of fresh medium containing sodium heparin (0.05 mg). Heparin-releasable LPL activity was measured by the method described previously (8). An aliquot (0.2 ml) of the medium was incubated with both 0.6 ml of emulsified glycerol tri[1-<sup>14</sup>C]oleate and 0.2 ml of diluted human serum for 60 min at 37°C. The amount of [1-<sup>14</sup>C]oleic acid degraded by LPL activity was extracted and counted with a scintillation counter. One unit of MLPLI was defined as follows; the activity added at the concentration of 1 unit/ml inhibits half of LPL activity expressed by control 3T3-L1 adipocytes.

**Purification Procedures:** Serum-free medium obtained (4.3 liters) was concentrated 8-fold by ultrafiltration under nitrogen using a Diaflo Cell

(type 8200) (Amicon Corp., Danvers, MA, USA) fitted with a Diafl YM-10 membrane (nominal mol wt cutoff, 10,000). The concentrates were dialyzed against 0.17 M acetic acid by using seamless cellulose tubing (Sanko Junyaku Co., Tokyo) at 4°C. The lyophilized sample was dissolved in 5 ml of 1 M acetic acid and the resulting supernatant (4.1 ml) was chromatographed on a column (2.0 x 57 cm) of Sephadex G-100 (Pharmacia, Uppsala, Sweden) which was equilibrated and eluted with 1 M acetic acid at the flow rate of 6 ml/hr at room temperature. Fractions of 2.3 ml each were collected. The fractions with the activity were finally purified by reverse-phase high-performance liquid chromatography (RP-HPLC) (model 130A, Applied Biosystems Inc., Foster City, CA, USA). The material was applied to an aquapore octyl RP-300 column (2.1 x 30 mm, Applied Biosystems Inc.) equilibrated with 0.1% trifluoroacetic acid (TFA), and then eluted on the conditions described in the figure legend.

**SDS Polyacrylamide Gel Electrophoresis (PAGE):** For SDS-PAGE, purified protein was dissolved in sample buffer containing 10% glycerol, 2.0% SDS and 5% 2-mercaptoethanol equilibrated to a pH of 6.8 with Tris. After being heated at 95°C for 5 min, the sample was electrophoresed in a 15% gel. The proteins were stained with silver using "Ag-stain DAIICHI kit" (Daiichi Kagaku Yakuhin Co., Tokyo).

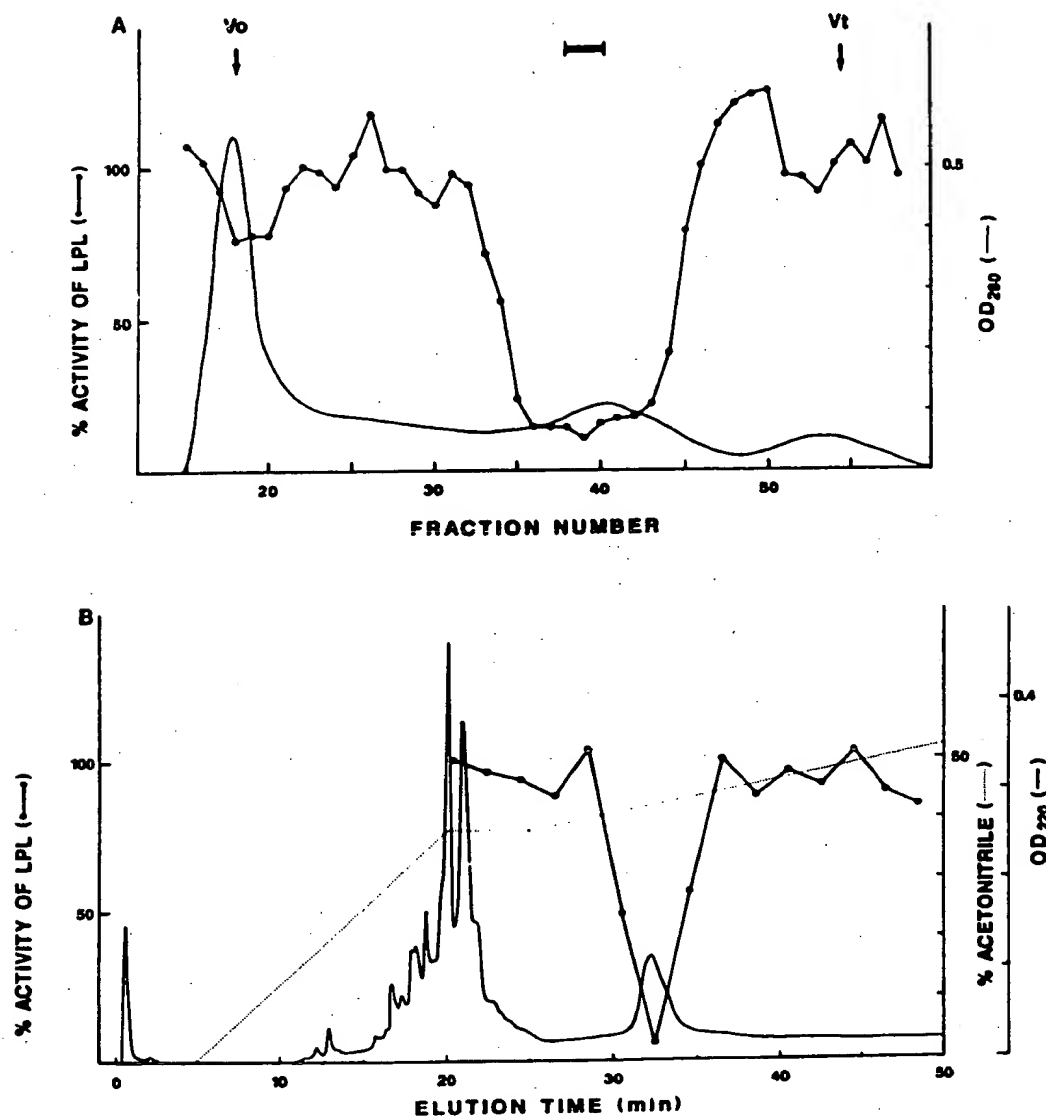
**Amino Acid Sequence Determination:** Amino acid sequence of the amino-terminal portion of the purified protein was determined by using a protein sequencer (model 477A, Applied Biosystems Inc.) equipped with an on-line PTH-analyzer (model 120A). Polybrene was used as a carrier.

**Protein Determinations:** Protein concentrations were determined by the method of Bradford (9) using a Bio-Rad dye-binding kit (Bio-Rad Laboratories, Richmond, CA, USA) at each step before RP-HPLC, with bovine serum albumin used as a standard. Since the protein content of fractions after the RP-HPLC step was low, it was determined from the absorbance profile.

### Results

**Cachexia Development after Transplantation of SEKI Tumor:** The nude mice bearing SEKI tumor developed marked weight loss and a cachexia-like appearance and then the condition progressed to death. On the 25th day after the transplantation, body weight decreased to  $73.6 \pm 8.95\%$  of those of control mice. This cachectic state completely disappeared within 10 days after resection of the tumor, with the recovery of the body weight to the control level.

**Isolation of LPL-inhibiting Factor from Cultured Medium of SEKI Cells:** The medium conditioned by SEKI cells inhibited heparin-releasable LPL activity of differentiated 3T3-L1 adipocytes in a dose-dependent manner, and it was calculated that 1 ml of the medium contained 160 units of MLPLI. By ultrafiltration, more than 60% of the activity was recovered in the concentrated solution. Gel filtration studies revealed that the activity was eluted in fractions from 36 to 43 (Fig. 1A). Fractions of 38, 39 and 40, which had potent activity, were applied to an RP-HPLC column (Fig. 1B). In the preliminary study the activity was eluted at about 42% acetonitrile concentration, so we set up an acetonitrile gradient as shown in Fig. 1B.



**Fig. 1.** Purification of LPL-inhibiting activity in the concentrated and dialysed medium. (A) Gel permeation chromatography. The points show the percentage of heparin-releasable LPL activity of 3T3-L1 adipocytes exposed to each fraction. The LPL activity expressed in control 3T3-L1 adipocytes was taken as 100%. The horizontal bar indicates the pooled fractions. The absorbance at 280 nm is indicated as the thin line. Fractions of 2.3 ml each were collected, lyophilized, reconstituted with 0.5 ml of 10 mM PES (pH 7.55), and then tested for LPL-inhibiting activity at a dilution of 1:7200.  $V_o$ , void volume.  $V_t$ , salt volume. (B) RP-HPLC. A sample (50  $\mu$ l) from the potent fractions in gel permeation chromatography was applied to an aquapore octyl RP-300 column equilibrated with 0.1% trifluoroacetic acid. The gradient (dotted line) consisted of 0-53% (v/v) acetonitrile at 36°C, with a flow rate of 0.2 ml/min. The column eluate was monitored at 220 nm (thin line). Fractions were removed at intervals of 2 min. A sample from each fraction was lyophilized, dissolved in 1 ml of 10 mM PBS and assayed at a dilution of 1:120 for LPL-inhibiting activity (points and thick line).

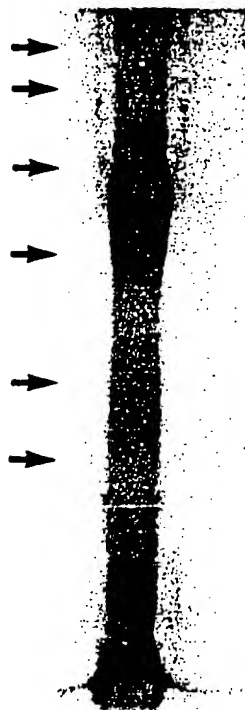


Fig. 2. SDS-PAGE of the purified MLPLI. The molecular weights of markers shown by arrows (from top to bottom) are 97,400, 66,200, 42,700, 31,000, 21,500, and 14,400, respectively. One major band with a molecular weight of 40 K-daltons was observed. Silver staining was used.

In this condition, LPL-inhibiting activity was eluted at 30-35 min. At the identical position, a single peak was detected by OD<sub>220</sub>, indicating that this protein is the substance responsible for the activity. The peak was collected and then used as purified melanoma-derived LPL inhibitor (MLPLI).

Partial Amino Acid Sequence of MLPLI: The purified MLPLI revealed a single peak by SDS-PAGE with a molecular weight of approximately 40 K-daltons (Fig. 2). The specific activity of purified MLPLI was calculated to be  $4.4 \times 10^6$  units/mg protein (Table 1). Amino acid sequence of the

Table 1. Purification steps and LPL-inhibiting activity

Purification steps	Total protein (μg)	Specific activity (units/mg protein)
Conditioned medium	$8.7 \times 10^4$	$8.0 \times 10^3$
Ultrafiltration and dialysis	$2.1 \times 10^4$	$2.0 \times 10^4$
Sephadex G-100	$6.0 \times 10^2$	$6.2 \times 10^5$
RP-HPLC	63	$4.4 \times 10^6$

MLPLI :

SPLPITPV-AT--IR-P

mature human LIF :

SPLPITPVNATCAIRHP

Fig. 3. Amino-terminal amino acid sequence of the purified MLPLI. That of mature human LIF is also shown. Hyphens indicate the positions of amino acids that were not determined in the present study.

amino-terminal portion was determined using about 170 pmol of the purified MLPLI. The protein was also considered to be in a pure form as estimated by the relative peak areas of PTH-amino acids liberated at each cycle. Similar results were obtained in 3 repeated experiments. Computer-aided analysis revealed that the amino-terminal sequence so far identified was completely identical to that of human leukemia inhibitory factor (LIF) as shown in Fig. 3 (10, 11).

#### Discussion

The reasons why we selected the SEKI cell line for the present study are given below. Kondo et al. examined 11 cancer cell lines for their ability to induce cancer cachexia in nude mice bearing these tumor cells, and found that only SEKI cell line could induce cachexia (12). Tamaoki and Ueyama observed that cachexia induced by this cell line is more severe than that of 264 other tumor cell lines transplantable to nude mice (Ken-ichi Tamaoki and Yoshihito Ueyama, personal communication). With respect to LPL-inhibiting activity, Kawakami et al. suggested that the conditioned medium of this cell line may possess the ability to inhibit LPL in cultured 3T3-L1 adipocytes (13). The present study demonstrates that the SEKI cell line used in our study can induce severe cachexia in nude mice and that the conditioned medium contains a factor with the ability to inhibit LPL in 3T3-L1 adipocytes. These results indicate that SEKI cell line preserved well the previously-reported biological characteristics related to cancer cachexia (12, 13).

We have temporarily designated this factor as melanoma-derived LPL inhibitor, MLPLI, and purified it. By ultrafiltration, gel filtration and RP-HPLC, MLPLI was purified to homogeneity. The molecular weight was estimated to be 40 K-daltons by SDS-PAGE. With respect to biological activity, it could be calculated that the concentrations of MLPLI effectively inhibiting LPL activity in 3T3-L1 adipocytes range 0.1 - 1.0 ng/ml; it is worth noting that this concentration is roughly comparable to that of TNF- $\alpha$ /cachectin (3), interleukin-1 (4) and interferon- $\gamma$  (5), when the same assay systems were used.

Several cytokines produced by host cells in response to the presence of cancer cells have the ability to inhibit LPL activity, and they are considered to participate in producing cancer cachexia (14). Our results indicate that purified MLPLI, a product of cancer cells, has a potent activity to inhibit LPL activity. Moreover, this factor was isolated from a human cancer cell line, SEKI, which induces cachexia in tumor-bearing nude mice. On the basis of these findings, it is reasonable to assume that MLPLI is a candidate for the factor responsible for cancer cachexia. To test this possibility, the effect of exogenously added MLPLI on experimental animals needs to be examined. Also, the relationship between MLPLI production and the development of cachexia should be investigated in a large number of human tumor xenografts in nude mice.

We further determined the amino acid sequence of MLPLI. Unexpectedly, 13 amino acids present at the amino-terminal portion of MLPLI are completely identical to those of LIF, suggesting that MLPLI purified in this study is a protein identical or closely related to LIF. LIF was originally isolated from cultured medium of Krebs II ascites cells by pursuing the biological activity, that is the induction of differentiation of the murine M1 myeloid leukemia cell line to macrophages (15). Two other factors isolated very recently were shown to be identical to LIF. One is human interleukin for DA cells; this cytokine supports proliferation of a murine interleukin-3-dependent leukemic cell line, DA-1a (16). The other is a polypeptide factor with activity to inhibit embryonic stem cell differentiation (17, 18). Further studies are required to determine whether MLPLI is identical to LIF or not.

#### Acknowledgments

The authors are grateful to Mr. K. Nagasaki for amino acid sequencing and Dr. N. Hirota for useful discussions. They also thank Dr. M. Sekiguchi for providing SEKI cell line. The present study was supported in part by a research grant from the Princess Takamatsu Cancer Research Fund, by a Grant-in-Aid from the Ministry of Health and Welfare for a Comprehensive 10-year Strategy of Cancer Control, by Grants-in-Aid for Cancer Research (61-1, 62S-1, 62-26) from the Ministry of Health and Welfare and by a Grant-in-Aid from the Mochida Memorial Foundation for Medical and Pharmaceutical Research.

#### References

1. Torti, F.M., Dieckmann, B., Beutler, B., Cerami, A., and Ringold, G.M. (1985) *Science* 229, 867-869.
2. Nilsson-Ehle, P., Garfinkel, A.S., and Schotz, M.C. (1980) *Ann. Rev. Biochem.* 49, 667-693.



3. Price, S.R., Olivecrona, T., and Pekala, P.H. (1986) Arch. Biochem. Biophys. 251, 738-746.
4. Beutler, B.A., and Cerami, A. (1985) J. Immunol. 135, 3969-3971.
5. Patton, J.S., Shepard, H.M., Wilking, H., Lewis, G., Aggarwal, B.B., Eessalu, T.E., Gavin, L.A., and Grunfeld, C. (1986) Proc. Natl. Acad. Sci. USA. 83, 8313-8317.
6. Rubin, C.S., Hirsch, A., Fung, C., and Rosen, O.M. (1978) J. Biol. Chem. 253, 7570-7578.
7. Shimoyama, M. (1975) In. In Vitro Culture of Human Cancer Cells (S. Oboshi, H. Sugano, Eds.) pp. 208-215, Asakura Shoten, Tokyo (in Japanese)
8. Iverius, P.-H., and Ostlund-Lindqvist, A.-M. (1986) Methods in Enzymology 129, 691-704.
9. Bradford, M.M. (1976) Anal. Biochem. 72, 248-254.
10. Gearing, D.P., Gough, N.M., King, J.A., Hilton, D.J., Nicola, N.A., Simpson, R.J., Nice, E.C., Kelso, A., and Metcalf, D. (1988) EMBO J. 6, 3995-4002.
11. Gough, N.M., Gearing, D.P., King, J.A., Willson, T.A., Hilton, D.J., Nicola, N.A., and Metcalf, D. (1988) Proc. Natl. Acad. Sci. USA. 85, 2623-2627.
12. Kondo, Y., Ueyama, Y., and Ohsawa, N. (1977) In. Proceedings of The Second International Workshop on Nude Mice. pp. 441-448, University of Tokyo Press, Tokyo.
13. Kawakami, M. (1985) Proceedings of the 27th Shishitu-Seikagaku Kenkyu 9-12. (in Japanese)
14. Beutler, B. (1988) Nutrition Reviews 46, 369-373.
15. Hilton, D.J., Nicola, N.A., Gough, N.M., and Metcalf, D. (1988) J. Biol. Chem. 263, 9238-9243.
16. Moreau, J.-F., Donaldson, D.D., Bennett, F., Witek-Giannotti, J., Clark, S.C., and Wong, G.G. (1988) Nature 336, 690-692.
17. Smith, A.G., Heath, J.K., Donaldson, D.D., Wong, G.G., Moreau, J., Stahl, M., and Rogers, D. (1988) Nature 336, 688-690.
18. Williams, R.L., Hilton, D.J., Pease, S., Wilson, T.A., Stewart, C.L., Gearing, D.P., Wagner, E.F., Metcalf, D., Nicola, N.A., and Gough, N.M. (1988) Nature 336, 684-687.